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Metal allergens nickel and cobalt facilitate TLR4 homodimerisation independently of MD2

Badrinarayanan Raghavan, Stefan F. Martin, Philipp R. Esser, Matthias Goebeler and Marc Schmidt

Corresponding author: Marc Schmidt, University of Giessen

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 01 June 2012

Thank you for your submission to EMBO reports. We have now received the enclosed two referee reports on it. As you will see, although the referees find the topic of interest, they also bring up some concerns regarding the conclusivenes of some of the results and their physiological relevance.

Given that both referees provide constructive suggestions on how to strengthen the study, I would like to give you the opportunity to revise your manuscript. In this case, as the novelty of the findings with respect to cobalt-induced allergies is not very high, all concerns would need to be addressed in full, to make the study as physiologically relevant as possible and strengthen the most novel finding -that of MD2 independent TLR4 homodimerisation. If all referee concerns are adequately addressed, we would be happy to consider your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

The length of revised manuscripts must be a maximum of 30,000 characters (including spaces, figure legends and references), and thus you will need to shorten the main text. Shortening may be made easier by combining the Results and Discussion into a single section, which we require, and which will help eliminate the redundancy that is inevitable when discussing the same experiments twice. In addition, although basic materials and methods essential to the understanding of the experiments must be described in the main body of the manuscript, more detailed explanations necessary to reproduce them may be presented as supplementary information. Please note that all information pertaining statistics must be retained in the figure legends.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The manuscript from Raghavan et al investigates the molecular mechanisms behind innate immune activation in cobalt derived contact hypersensitivity. By comparing inflammatory responses of different relevant cell types in the skin, they determine that cobalt responsive cells e.g. endothelial cells and dendritic cells express the pattern recognition receptor TLR4 while the unresponsive keratinocytes do not. The findings in keratinocytes in this paper are contrary to some earlier literature, but these concerns are adequately discussed and addressed in supplemental figures. The exogenous expression of TLR4 and MD2 in HEK293 cells confers them with responsiveness to cobalt. Further co-immunoprecipitation experiments show that both cobalt and nickel induce TLR4 dimerization and activation through interaction with two key histidine residues and that these histidines are specific for the metal reaction and not for an LPS response. Finally they show that expression of a soluble, truncated form of TLR4 is able to block the response to Cobalt and Nickel but not the LPS response. The manuscript is a direct follow on from the previously published paper identifying the interaction of TLR4 with nickel, and while the mechanism behind Co induced inflammation has not been addressed before, the findings are not particularly novel.

Crucial concerns:

1. TLR4 -mediated recognition of cobalt has been confirmed by over-expression of hTLR4 and hMD2 in cells lacking the endogenous receptors. However, hTLR4 and hMD2 should also be knocked down/out e.g. siRNA in cells that do normally express the receptors such as HUVECs and Thp-1s, and loss of reactivity to cobalt confirmed
2. Figure 2C: the levels of IL-8 are too low to even be detected by the ELISA kit. Unless there is an error and the IL-8 is actually in ng/ml rather than pg/ml, then I would consider this data to be interpretable as the levels are too low as to show real responsiveness being conferred.
3. Figure 4: The hTLR4-Q628* should also be shown to have this specificity in relevant immune/endothelial cell types. Thus experiments showing that hTLR4 - Q628* also blocked Co2+ and Ni2+ induced IL-8 in HUVECs, DCs or Thp-1s but not the LPS response.
4. Similarly for Supporting Information Figure 2C, the dose response of Cobalt in HEK293 expressing hTLR4/hMD2, the IL-8 levels are in the far low detection range for the ELISA. In other figures these cells produce up to 400pg/ml IL-8 with the same dose of cobalt chloride. While there can be variation between experiments, there shouldn't be a variation of a factor of 10?

Referee #2:

This potentially very interesting MS represents a "follow up" of a recent report (2010) describing a crucial role for human(h)TLR4 in the development of contact allergy to nickel(Ni). Based on mutagenesis data the autors concluded that two Ni ions bind to a histidine triplet provided by two opposing hTLR4 molecules thus initiating TLR4 homodimerisation.
Here the authors first extend this concept to cloely related Cobald(Co)ions. While DCs and endothelial cells are shown to respond to Co ions via production of proinflammatory cytokines keratinocytes fail to do so due to a lack of TLR4. As reported for Ni ions also Co ions activated

species-dependently TLR4.Intersting co-immunoprecipitation data lead the the authors to conclude that both Ni and Co ions facilitate hTLR4 homodimerisation independent of MD2 - yet the latter is clearly required for cytokine production.Based on recent cristal TLR4 data of Park rt al. the autors provide novel and potentially most exiting data showing that transfected h TLR4-N433A depict a gradual loss of HTLR4 homodimerisation - and subsequent Il8 production - and that soluble TLR4 inhibit NI/Co induced NFkB activation.
While personally I tend to accept the novel and exiting conclusions drawn, there are minor technical problems in Fig3 that need to be overcome.In Fig 3A anti Flag IPs bring down (without stimulation) visible HA- tagged TLR4 (rated as positiv - yet in Fig3B (Hek 293 WT cells)the same faint intensity caused by LPS is rated as negativ. Why is in Fig3C the staining intensity of co-precipitated HA-tagged TLR4 so high when compared to that of Fig3A/B? (compare Fig2A with Fig.3C)
Furthermore the authors need to discuss their observation that MD2 is required for cytokine production yet dispensible for Ni/Co induced TLR4 homodimerisation - where is the "bottle neck"?

1st Revision - authors' response 21 August 2012

Referee #1:

The manuscript from Raghavan et al investigates the molecular mechanisms behind innate immune activation in cobalt-derived contact hypersensitivity. By comparing inflammatory responses of different relevant cell types in the skin, they determine that cobalt responsive cells e.g. endothelial cells and dendritic cells express the pattern recognition receptor TLR4 while the unresponsive keratinocytes do not. The findings in keratinocytes in this paper are contrary to some earlier literature, but these concerns are adequately discussed and addressed in supplemental figures. The exogenous expression of TLR4 and MD2 in HEK293 cells confers them with responsiveness to cobalt. Further co-immunoprecipitation experiments show that both cobalt and nickel induce TLR4 dimerization and activation through interaction with two key histidine residues and that these histidines are specific for the metal reaction and not for an LPS response. Finally they show that expression of a soluble, truncated form of TLR4 is able to block the response to Cobalt and Nickel but not the LPS response. The manuscript is a direct follow on from the previously published paper identifying the interaction of TLR4 with nickel, and while the mechanism behind Co induced inflammation has not been addressed before, the findings are not particularly novel.

Crucial concerns:

1. TLR4 -mediated recognition of cobalt has been confirmed by over-expression of hTLR4 and hMD2 in cells lacking the endogenous receptors. However, hTLR4 and hMD2 should also be knocked down/out e.g. siRNA in cells that do normally express the receptors such as HUVECs and Thp-1s, and loss of reactivity to cobalt confirmed

We now additionally have performed siRNA experiments, in which we knocked down MD2 and TLR4 in human primary ECs. These new data confirm requirement of both MD2 and TLR4 for cobalt-induced proinflammatory gene expression and have now been integrated into Fig. 2 as subfigure D.

2. *Figure 2C: the levels of IL-8 are too low to even be detected by the ELISA kit. Unless there is an error and the IL-8 is actually in ng/ml rather than pg/ml, then I would consider this data to be interpretable as the levels are too low as to show real responsiveness being conferred.*

We thank the referee for this comment and apologize for this error. Supernatants were each used in a 1:100 dilution to measure IL-8. However, due to a replacement of our old ELISA-Reader by a new machine that uses software that no longer automatically counts in the dilution factor, the IL-8 concentration values in the original figure 2C by mistake were given in a dimension 100-fold too low. This error has now been corrected in the revised Fig 2C, in which we not only corrected the wrong unit designation of the original figure but now show the results of new IL-8 ELISA experiments, in which the original supernatents were remeasured at lower dilution (1:10) to avoid accuracy issues arising from low raw value counts

too close to the manufacturer-specified detection limit of 0.8 pg/ml IL-8 for the employed ELISA system.

3. *Figure 4: The hTLR4-Q628* should also be shown to have this specificity in relevant immune/endothelial cell types. Thus experiments showing that hTLR4 - Q628* also blocked Co2+ and Ni2+ induced IL-8 in HUVECs, DCs or Thp-1s but not the LPS response.*

As suggested, we now have tested the effect of hTLR4-Q628* (sTLR4) on the responsiveness of HUVEC to nickel, cobalt and LPS. Analogous to the HEK293-TLR4/MD2 cell line, presence of sTLR4 inhibited metal- but not LPS-dependent IL-8 production in these primary cells. The new data are now provided as additional Fig 4C.

4. *Similarly for Supporting Information Figure 2C, the dose response of Cobalt in HEK293 expressing hTLR4/hMD2, the IL-8 levels are in the far low detection range for the ELISA. In other figures these cells produce up to 400pg/ml IL-8 with the same dose of cobalt chloride. While there can be variation between experiments, there shouldn't be a variation of a factor of 10?*

Similar to Fig. 2C (see point #2), in the initial Supporting Figure 2C diluted supernatants have been used to determine IL-8 levels in the respective samples but by mistake the dilution factor of 1:10 has been neglected in the initial calculations. This error has now been corrected in the revised Supplementary Fig. S2C, which now shows the correct values.

Regarding the criticized variation of total values across different experiments with a given cell line, we would like to state that for the different experiments shown in separate subfigures culture dish sizes and seeding densities were different (e.g. in Supplementary Fig.S2B cells were stimulated in 10 cm dishes, whereas for the experiments shown in Fig. S2C and D cells were grown and stimulated in 96 well formats but stimulated at different days after seeding resulting in variable total values across the individual experiments). Due to space restrictions seeding formats and densities are not explicitely mentioned in the legends. However, we would like to point out that plate size, seeding density and timing of the stimulation were strictly maintained for reproduction of individual experiments to exclude wrong interpretations due to the use of different well formats and different cell densities. Furthermore, we now carefully checked all ELISA-derived values again for potentially wrong calculations resulting from disregarded dilution factors. Accordingly, we corrected a previously undetected error in Fig. 1B.

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This potentially very interesting MS represents a "follow up" of a recent report (2010) describing a crucial role for human(h)TLR4 in the development of contact allergy to nickel (Ni). Based on mutagenesis data the autors concluded that two Ni ions bind to a histidine triplet provided by two opposing hTLR4 molecules thus initiating TLR4 homodimerisation.
Here the authors first extend this concept to closely related Cobalt (Co)ions. While DCs and endothelial cells are shown to respond to Co ions via production of proinflammatory cytokines keratinocytes fail to do so due to a lack of TLR4. As reported for Ni ions also Co ions activated species-dependently TLR4. Interesting co-immunoprecipitation data lead the the authors to conclude that both Ni and Co ions facilitate hTLR4 homodimerisation independent of MD2 - yet the latter is clearly required for cytokine production. Based on recent crystal TLR4 data of Park et al. the autors provide novel and potentially most exiting data showing that transfected hTLR4-N433A depict a gradual loss of hTLR4 homodimerisation - and subsequent IL8 production - and that soluble TLR4 inhibit Ni/Co induced NFkB activation.

1. While personally I tend to accept the novel and exiting conclusions drawn, there are minor technical problems in Fig 3 that need to be overcome. In Fig 3A anti Flag IPs bring down (without stimulation) visible HA- tagged TLR4 (rated as positiv - yet in Fig3B (Hek 293 WT cells) the same faint intensity caused by LPS is rated as negativ.

What we meant to say was that in Figure 3B LPS did not increase dimerisation above background. To clarify this, we rephrased our initial statement on page 7, paragraph 3, lines 10-12 of the final manuscript and now state: "Unlike LPS, however, which did not raise

dimerisation above background both metal allergens also facilitated complex formation in absence of MD2 (Fig 3B).".

2. *Why is in Fig3C the staining intensity of co-precipitated HA- tagged TLR4 so high when compared to that of Fig3A/B? (compare Fig3A with Fig.3C)?*

We initally chose to show an extremely long exposure of the film in Fig. 3C to illustrate gradual loss of dimerization capacity upon single and double mutation of N433, leading to an apparently strong dimerization band in the unstimulated samples. While we do not deny the appearance of a background dimerisation band in absence of stimulation (as we explicitly state in the discussion of Fig. 3A), the shown strong band may lead to overinterpretation of this observation, even more since other repetitions of this experiment did not reveal presence of such an excessive background band. We thus decided to repeat the experiment in Fig. 3C once again and now provide a better and more representative blot to support our initial conclusions.

3. *Furthermore the authors need to discuss their observation that MD2 is required for cytokine production yet dispensible for Ni/Co induced TLR4 homodimerisation - where is the "bottle neck"?*

TLR4 and MD2 are both crucial for initiation of proinflammatory gene expression by Co and Ni as also supported by the newly included siRNA data in Fig 2D. However, while TLR4 directly participates in metal binding, MD2 is dispensable for metal-induced TLR4 dimerisation. One possible interpretation is that MD2 binding may subsequently be required to allow appropriate signaling from the Co/Ni-bound TLR4 dimer. Thus, MD2 may additonally shape the complex to yield an active confirmation that allows intracellular signaling to occur. This speculation has now been added on page 8, paragraph 3 lines 5-8, where we state: "Since MD2 was clearly indispensable for metal-induced proinflammatory gene expression MD2 may perhaps serve to further shape the metal-bound TLR4 receptor complex to allow effective signal initiation."

2nd Editorial Decision 21 September 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. We sincerely apologize for the delay in sending you our final decision. Thank you for your contribution to our journal.

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Yours sincerely,

Editor
EMBO Reports

REFEREE REPORT:

Referee #2

In its amended version the authors not only fully answer in detail questions of the referees but also correct some mistakes (calculation of IL8 concentration) and add new information (loss of responsivness upon si RNA mediated suppression of IL4) In its present form the MS significantly forsters our understanding of Ni and Co mediated TLR4 homodimerisation as basis of respective contact allergy.

